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Comparison of in vivo cholinesterase inhibition in neonatal and adult rats by three organophosphorothioate insecticides*

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Summary

Developing mammals are more sensitive than adults to a variety of organophosphorothioate insecticides (OPs), compounds which act in vivo by inhibition of cholinesterase (ChE). Little is known, however, regarding age-related differences in biochemical responses to these toxicants. The time course of ChE inhibition and recovery in whole brain was compared in neonatal (7 days of age) and adult (80—100 days of age) rats after treatment with maximal tolerated doses (MTDs) of either methyl parathion (MPS), parathion (PS) or chlorpyrifos (CPF). Neonatal rats were more sensitive than adults in all cases (MTDs for MPS, PS and CPF: neonates = 7.8, 2.1 and 45 mg/kg, s.c.; adults = 18, 18, and 279 mg/kg, s.c., respectively). In general, maximal brain ChE inhibition was similar (>78%) in both age groups but ChE activity recovered faster in neonates. Plasma and erythrocyte ChE activities correlated relatively well (r = 0.794—0.943) with brain ChE activity in neonatal rats at all time points between 4 h and 7 days after treatment but similar correlations between circulating and brain ChE activities in adults were more variable (r = 0.211—0.917). The results indicate that neonatal rats are more sensitive to acute lethality from these compounds and that MTD exposures produce extensive brain ChE inhibition in both age groups. Significant inhibitor-related and age-related differences in the duration of ChE inhibition can ensue, however, following such OP exposures.

Key words: Organophosphate; Developmental; Cholinesterase inhibition; Parathion; Methyl parathion; Chlorpyrifos

Introduction

Organophosphorus insecticides (OPs) are used extensively throughout the world to control agricultural and urban insect pests [1,2]. These compounds exert acute toxicity in target organisms through inhibition of acetylcholinesterase, causing accumulation of acetylcholine at synaptic terminals and excessive stimulation of postsynaptic cells [2]. Death from exposure to OPs is considered to be primarily the

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consequence of overstimulation of central respiratory centers, with subsequent failure of ventilatory drive [3,4].

Of particular concern are possible effects of exposure to anticholinesterases during development. Previous investigators have noted that young mammals are generally more sensitive to acute toxicity (using lethality as an endpoint) from a variety of OPs [5—8]. These age-related differences in acute response to OPs appear at least partially due to ontogenetic differences in biotransformation [5,8]. While the sensitivity of brain ChE to inhibition in vitro by OPs was examined (as a possible mechanism for age-related differences in sensitivity) and found not to change during postnatal development [5], no comparisons of in vivo biochemical responses to OPs have been reported between age groups showing marked differences in sensitivity. The present study was performed to compare the responses of young (7 days) and adult (80—100 days) rats to exposure to three common OP insecticides — methyl parathion, parathion and chlorpyrifos.

Methods

Animals

Sprague—Dawley rats (purchased from Harlan Sprague—Dawley, Indianapolis, IN) were used throughout. Pregnant females were caged individually and birth dates recorded. Pups were routinely randomized between dams and culled to eight pups/dam (no selection regarding sex) on postnatal day 2. Adult male rats were raised in community cages until one week before treatment, at which time they were housed individually in steel mesh cages. Animals were reared under a 12 h light:12 h dark illumination cycle. Mean body weights (± S.E.M.) at time of treatment were 14.5 ± 0.2 g for neonates and 376.7 ± 2.9 g for adults.

Chemicals

Methyl parathion (*O,O'*-dimethyl-*p*-nitrophenyl phosphorothioate), parathion (*O,O'*-diethyl-*p*-nitrophenyl phosphorothioate), and chlorpyrifos (*O,O'*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate) were purchased from Chem Service (West Chester, PA) and were at least 98% purity. [^{3}H]Acetylcholine iodide (specific activity, 100 mCi/mmol) was purchased from New England Nuclear, Boston, MA. All other chemicals were reagent grade.

Dosing and determination of maximal tolerated doses (MTDs)

OP compounds were dissolved in peanut oil and injected subcutaneously at an injection volume of 1 ml/kg body weight (except in the case of chlorpyrifos treatment in adults in which an injection volume of 2 ml/kg was used). Maximal tolerated doses (MTDs) were established by first estimating the LD_{50} for each compound from available literature and then deriving a series of doses by either multiplying or dividing the LD_{50} estimate by a factor of 1.3 [9]. For each inhibitor, rats (7 days of age for neonates, 80—100 days of age for adults; n = 6/dose) were treated with one of these doses and signs of toxicity and cumulative lethality recorded over a seven day period. The highest dose level which caused no lethality in this preliminary study was the estimate of the maximal tolerated dose (MTD_{est}). A second lethality experiment was then performed using doses of MTD_{est}, $MTD_{est}/1.3$ and $MTD_{est}/1.69$ (n =

10/dose) and toxicity and lethality observed for 7 days. The highest dose from this second study in which no lethality was observed was defined as the maximal tolerated dose (MTD) and was used for examination of the time course of in vivo ChE inhibition and recovery.

Time course of ChE activity following MTD treatment

Rats (n = 5—6/time point) were treated with the MTD for one of the three OP compounds (methyl parathion: adults = 18 mg/kg, neonates = 7.8 mg/kg; parathion: adults = 18 mg/kg, neonates = 2.1 mg/kg; chlorpyrifos: adults = 279 mg/kg, neonates = 45 mg/kg) and sacrificed at various times after treatment for determination of ChE activity in whole brain, plasma and erythrocytes. Animals were anesthetised with diethyl ether and blood obtained by cardiac puncture (in heparinized syringes), after which the rat was decaptitated and whole brain was rapidly dissected and frozen at −55°C until assay. Whole heparinized blood was centrifuged for 1 min in a microcentrifuge (Beckman Microfuge B, Beckman Instruments, Fullerton, CA), and plasma withdrawn and frozen. A 0.05 ml sample of packed erythrocytes was washed in 1 ml of saline, centrifuged for 1 min as before, the supernatant was discarded and then washed cells were frozen until assay.

Biochemical assays

Plasma and erythrocyte samples were thawed at room temperature and diluted (1:4, v:v) with 50 mM potassium phosphate buffer, pH 7.0 (phosphate buffer) before assay. Whole brain was thawed and homogenized in 9 volumes phosphate buffer (1:10, w:v) and then homogenates were diluted another five-fold before assay (1:50 final dilution). Cholinesterase activity was measured radiometrically by the method of Johnson and Russell [10] using [^{3}H]acetylcholine iodide as substrate (0.12 mM final concentration). Each reaction vial (0.1 ml final volume) contained 0.6% Triton X-100 to aid in membrane disruption. Protein content of the brain samples was estimated by the method of Lowry and co-workers [11] using bovine serum albumin as standard. Cholinesterase activity was reported as cpm/min per μl tissue (plasma and erythrocytes) or cpm/min per μg protein (brain) and plotted as percent of control activity as a function of time after treatment.

Data analysis

Differences in brain ChE inhibition observed between age groups for each inhibitor at the maximal tolerated dose were analyzed by analysis of variance using the SAS General Linear Models (GLM) program [12]. Effects of pesticide treatment on body weight were examined 7 days after treatment by one way analysis of variance using the SAS GLM program. Correlation coefficients (r values) for within-subject comparisons of brain ChE activity with either plasma or erythrocyte ChE activity at all times after treatment were determined using the SAS REGression procedure [15].

Results

Acute toxicity

Table I shows maximal tolerated doses (MTDs) for the three OP insecticides in

TABLE I

MAXIMAL TOLERATED DOSES IN NEONATAL AND ADULT RATS

Treatment[a]	Maximal tolerated dose (mg/kg)	
	Neonate	Adult
Methyl parathion	7.8	18
Parathion	2.1	18
Chlorpyrifos	45.0	279[b]

[a]OP compounds were dissolved in peanut oil and graded doses administered subcutaneously in an injection volume of 1 ml/kg body weight. Cumulative lethality was recorded for 7 days after treatment and maximal tolerated dose (highest dose resulting in 0% lethality, $n = 16$) determined as in Methods.
[b]Chlorpyrifos was dissolved in peanut oil such that injection volume was 2 ml/kg body weight.

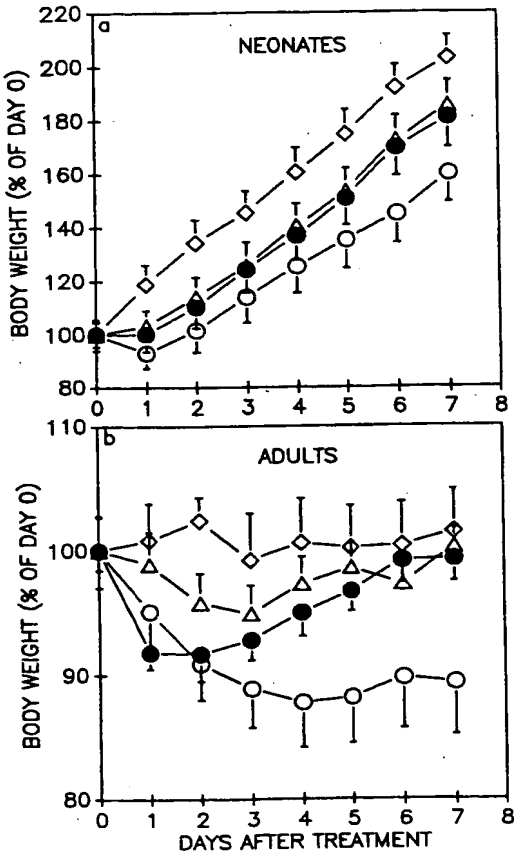

Fig. 1. Body weights in neonatal and adult rats after treatment with maximal tolerated doses of either methyl parathion, parathion or chlorpyrifos. (a) Neonatal rats (7 days of age) were treated with either peanut oil (1 ml/kg, s.c., ◇) or methyl parathion (7.8 mg/kg, ●), parathion (2.1 mg/kg, ○) or chlorpyrifos (45 mg/kg, △) in peanut oil. Body weights were recorded daily and plotted as percent of weight on day of injection (mean ± S.E.M.). (b) Adult male rats (80—100 days of age) were treated with either peanut oil (1 ml/kg, s.c.) or methyl parathion (18 mg/kg), parathion (18 mg/kg) or chlorpyrifos (279 mg/kg, 2 ml/kg) and body weights recorded daily and plotted as percent of weight on day of injection (mean ± S.E.M.). Symbols for treatment groups same as above.

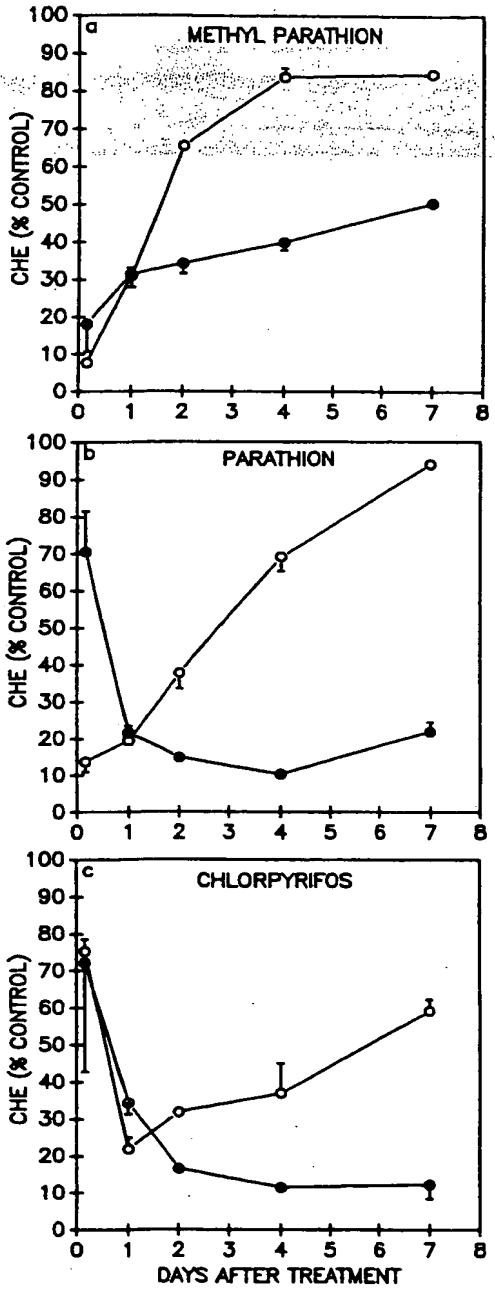

Fig. 2. Brain cholinesterase (ChE) activity in neonatal (o) and adult (●) rats at various times after maximal tolerated doses (MTDs) of either (a) methyl parathion, (b) parathion or (c) chlorpyrifos. Rats were treated, sacrificed at various times after treatment and ChE activity measured as described in Methods. Values represent mean cpm [^{3}H]acetylcholine hydrolyzed/min per μg protein ± S.E.M. and are plotted as percent of control.

neonatal (postnatal day 7) and adult male (80—100 days of age) rats. In all cases, the OPs were more potent toxicants in neonates, causing lethality at considerably lower doses than in adults. Few signs of overt toxicity were noted in neonatal rats treated with MTDs: "apparent" tremors were occasionally observed in OP-treated animals but typical signs of "cholinergic crisis" were not noted. In general, adult rats treated with maximal tolerated doses of either parathion, methyl parathion or chlorpyrifos showed no signs or only slight to moderate signs of acute toxicity (e.g., diarrhea, fasciculations, lacrimation, slight tremors).

Figure 1 shows the effects of single maximal tolerated doses on body weights in neonatal and adult rats. Neonates treated with maximal tolerated doses of either methyl parathion, parathion or chlorpyrifos showed a transient decrease in body weight gain over approximately the first day after treatment, after which relatively similar rates of weight gain occurred over the next 6 days of observation (Fig. 1a). Adult rats treated with the MTD of either methyl parathion or chlorpyrifos also showed a transient decrease ($< 10\%$) in body weight which recovered within 7 days (Fig. 1b). Adults treated with parathion however, showed a greater reduction (10—12%) in body weight with no evidence of recovery to control values by 7 days after treatment. Parathion treatment caused a significant ($P < 0.04$) reduction in body weight in both age groups at the end of the 7-day observation period.

Cholinesterase inhibition

Figure 2 shows the time course of inhibition and recovery of brain ChE activity in neonatal and adult rats following exposure to maximal tolerated doses of each of the three pesticides. Control neonatal brain ChE activity increased approximately 42% from 7 to 14 days of age (mean ± S.E.M. = 140 ± 3 to 199 ± 8 cpm [^{3}H]acetylcholine hydrolyzed/min per μg protein at 7 and 14 days, respectively) whereas control adult values remained relatively constant during the 7 days of observation (mean ± S.E. = 238 ± 4 cpm [^{3}H]acetylcholine hydrolyzed/min per μg protein). Methyl parathion (18 mg/kg), parathion (18 mg/kg) or chlorpyrifos (279 mg/kg) produced 82—89% maximal brain ChE inhibition in adults. Relatively similar degrees (79—92%) of maximal brain ChE inhibition were noted in neonates treated with the respective MTDs for methyl parathion (7.8 mg/kg), parathion (2.1 mg/kg) or chlorpyrifos (45 mg/kg). In all cases, brain ChE activity recovered faster in neonates compared to adults. Analysis of the inhibition profiles indicated a significant age-related difference in brain ChE inhibition over time with all three compounds (parathion, $P < 0.02$; methyl parathion, $P < 0.005$; chlorpyrifos, $P < 0.03$).

Figures 3 and 4 show the inhibition of plasma and erythrocyte ChE by the respective MTDs. Minimal recovery of either plasma or erythrocyte ChE activities was noted in adults during the 7 days following treatment (except for recovery of plasma ChE after methyl parathion exposure, Fig. 3a) whereas both plasma and erythrocyte ChE showed evidence of significant recovery in neonates. Table II shows the linear correlation coefficients when ChE activities in either the plasma or erythrocyte fractions were compared with residual activity in whole brain at all times between 4 h and 7 days after treatment. Inhibition of both plasma and erythrocyte ChE activity in neonates correlated better with brain activity ($r = 0.794$—0.943) than those same comparisons in adults ($r = 0.211$—0.917). The only nonsignificant ($P > 0.05$, analy-

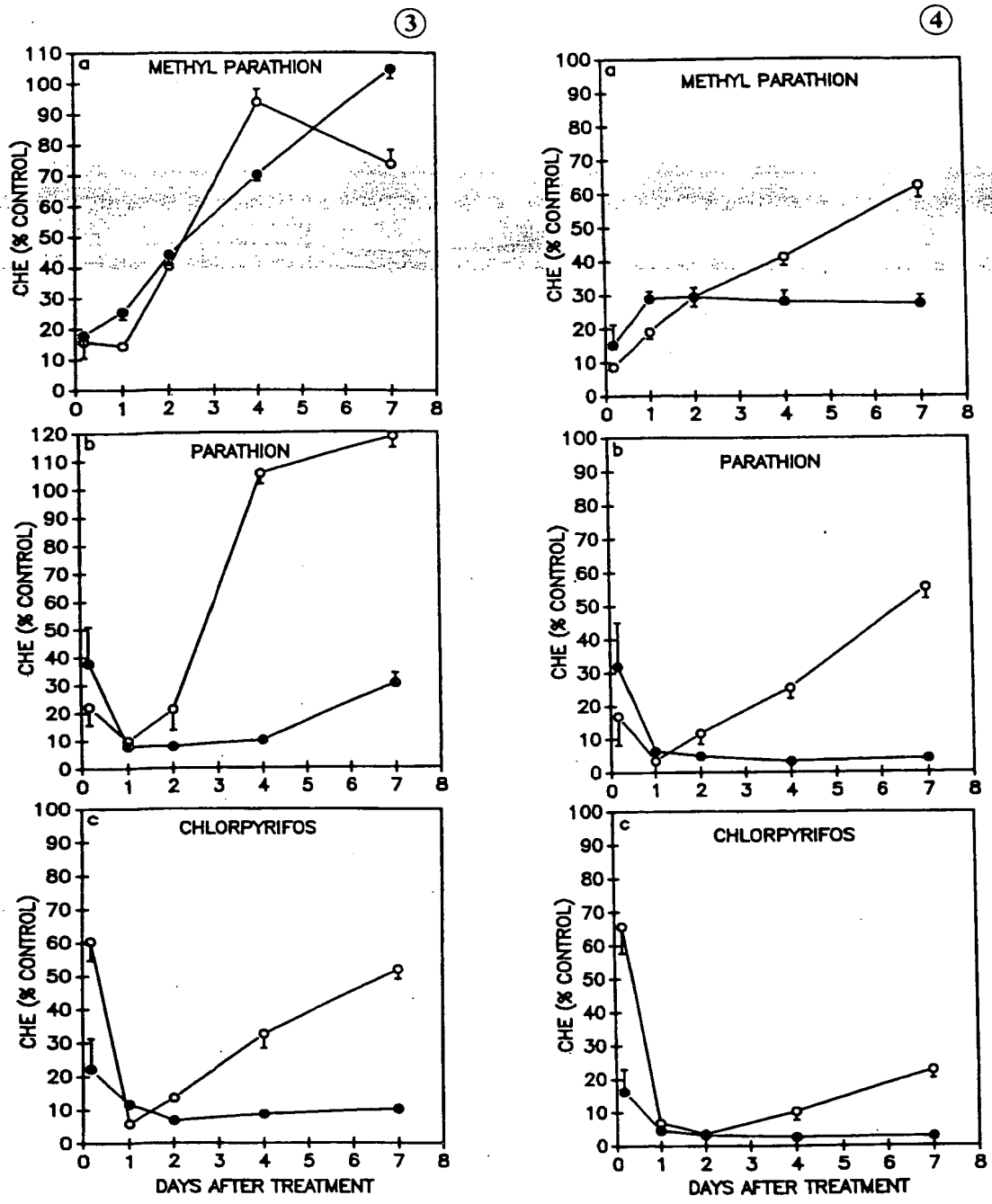

Fig. 3. Plasma ChE activity in neonatal (o) and adult (•) rats at various times after maximal tolerated doses of either (a) methyl parathion, (b) parathion, or (c) chlorpyrifos. Values represent mean cpm [^{3}H]acetylcholine hydrolyzed/min per μl plasma ± S.E.M. and are plotted as percent of control.

Fig. 4. Erythrocyte ChE activity in neonatal (o) and adult (•) rats at various times after maximal tolerated doses of either (a) methyl parathion, (b) parathion or (c) chlorpyrifos. Values represent mean cpm [^{3}H]acetylcholine hydrolyzed/min per μl erythrocytes ± S.E.M. and are plotted as percent of control.

TABLE II

CORRELATION OF BRAIN CHE WITH PLASMA OR ERYTHROCYTE CHE

Treatment	Correlation coefficients[a]			
	Brain vs. plasma		Brain vs. RBC	
	Neonate	Adult	Neonate	Adult
Methyl parathion	0.861	0.842	0.858	0.211
Parathion	0.943	0.565	0.794	0.764
Chlorpyrifos	0.940	0.367	0.809	0.917

[a]Correlation coefficients (r values) were determined by plotting residual brain ChE activity versus residual plasma or erythrocyte (RBC) ChE activity at all time points between 4 h and 7 days after treatment with maximal tolerated doses of either parathion, methyl parathion or chlorpyrifos.

sis of variance) correlation noted was the comparison between brain ChE and erythrocyte ChE in adult rats treated with methyl parathion (see Table II).

Discussion

As suggested by previous studies [5—8], neonatal rats were more sensitive than adults to the acute toxicity of three common organophosphorus insecticides; maximal tolerated doses (MTDs) indicated that neonates were approximately 2.3, 8.6 and 6.2 times more sensitive than adults to the acute toxicity of methyl parathion, parathion and chlorpyrifos, respectively. In the case of methyl parathion and parathion, the ontogenetic development of a variety of enzymatic and nonenzymatic detoxification processes (e.g., "A"-esterase, glutathione-dependent dealkylation and dearylation, and binding to "nonvital tissue constituents") appears related to the age-related differences in toxicity [5,8]. The MTDs for all three pesticides observed in adult rats were comparable or higher than reported LD_{50} values for these same compounds when administered by the oral or intraperitoneal route [5,13,14]. This may be a reflection of substantially slower absorption after subcutaneous administration of these pesticides compared to other routes.

Acute exposure to maximal tolerated doses of any of the three OP insecticides caused extensive inhibition of ChE activity in brain, plasma and erythrocytes in both age groups of rats. Methyl parathion, parathion and chlorpyrifos caused similar levels ($>78\%$) of maximal brain ChE inhibition in both age groups but esterase activity recovered more rapidly in neonates. The age-related differences in the persistence of ChE inhibition in the nervous system was expected due to the rapid rate of protein synthesis in the developing brain compared to adults [15], but may also be a function of the larger doses administered to adults. Alternatively, because the doses used were chosen on the basis of being just below the "threshold" for lethality,

another possibility is that neonatal rats respond similarly as adults to certain levels of acute brain ChE inhibition, but are more sensitive to prolonged depression of ChE which would occur with higher doses.

Differences in ChE recovery among the three OP inhibitors were probably related to differences in absorption, biotransformation and/or ChE reactivation. The more rapid reappearance of brain ChE activity in adult rats treated with methyl parathion compared to that seen following parathion exposure (Fig. 2) is probably related to the more rapid spontaneous reactivation of dimethylphosphorylated ChE relative to diethylphosphorylated enzyme [16]. In contrast, rates of spontaneous deacylation should be similar after inhibition of ChE by either parathion or chlorpyrifos as the diethylphosphorylated enzyme is identical, regardless of the nature of the "leaving" group originally on the OP molecule. Therefore, more rapid recovery of brain ChE activity in neonates following parathion exposure compared to that following chlorpyrifos treatment may be due to protracted absorption of chlorpyrifos from the subcutaneous injection site.

Overwhelming evidence supports a role for acetylcholinesterase in neuronal function through the regulation of acetylcholine levels at synaptic terminals. In contrast, ChE activity in either plasma or erythrocytes has no known physiological function. It is generally accepted however, that these circulating enzymes can be used as readily obtainable, reliable markers for detection of exposure to cholinesterase inhibitors. When the residual ChE activity in plasma was compared with ChE activity in brain at various times after treatment with OPs, relatively good correlations were obtained in neonates (r = 0.861—0.943). Similar correlations between erythrocyte ChE and brain ChE in neonates were not as high (r = 0.794—0.858) as between plasma and brain. Correlations between blood ChE activities and brain ChE in adults were highly variable (r = 0.211—0.917). These data suggest that, under certain conditions, circulating ChE activities may be experimentally useful in estimating the extent of brain ChE inhibition following OP exposures.

While it is apparent that young animals can be more sensitive to the lethal effects of high-level exposures to these compounds, little is known regarding the susceptibility of the developing nervous system to low-level OP exposures. Most mammals undergo extensive postnatal development within the central nervous system: in rodents, the "brain growth spurt" occurs from birth through about the fourth postnatal week. During this period, extensive increases in neurochemical markers for cholinergic neurons occur in the brain, coinciding with maturation of synaptic connections [17—19]. In addition, several studies suggest that ChE may have a role in neuronal development [20—23]. Inhibition of cholinesterase in the nervous system during this critical period may therefore disrupt cellular processes of growth and differentiation and alter normal brain cytoarchitecture. Stamper and co-workers [24] demonstrated downregulation of cholinergic receptors and deficits in spatial memory of rats treated daily (postnatal day 5—20) with parathion (1.3 or 1.9 mg/kg per day). Veronesi and Pope [25] reported neurochemical and histological changes indicative of altered hippocampal circuitry following parathion exposure in neonatal rats (0.882 mg/kg per day, postnatal day 5—20). These studies suggest that early postnatal exposure to OPs may produce persistent changes in the developing nervous system.

Recently, public attention has focused on the possibility that young children may be more sensitive than adults to neurotoxic effects of pesticide residues in food, both because of inherent differences in sensitivity and because of differences in diet which amplify relative pesticide exposures in infants [26]. While the extent of food-borne pesticide intake in young children may have been grossly overestimated in that report [27], the inherent age-related differences in sensitivity to the acute toxicity (lethality) from OPs suggest a closer examination of low-level exposures during development is warranted. Knowledge of the inhibitor-dependent and time-dependent effects of OPs on brain ChE will aid in the assessment of comparative neurotoxicity in developing and adult nervous systems from anticholinesterase exposures.

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